Dossier

In vivo potentiation of 5-fluorouracil by leucovorin in murine colon carcinoma

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Summary — In vitro results have clearly demonstrated that leucovorin (LV) can enhance the growth inhibitory effects of 5-fluorouracil (SFU) but in vivo potentiation of the antitumor effect of SFU by LV has not yet been defined in animal models. The antitumor effect and the toxicity of the LV-SFU combination was studied in mice bearing the colon carcinomas, Colon 26 and Colon 38. Mice were treated weekly with SFU at the maximum tolerated dose (100 mg/kg) alone or with LV at different doses and schedules. Pretreatment with LV followed 1 hr later by a second LV injection together with SFU clearly potentiated the antitumor effect of SFU in both murine tumor lines. Comparable results were obtained with total LV doses of 100 and 200 mg/kg. The effect of SFU pretreatment was studied by randomization of 5FU pretreated Colon 38-bearing mice in 2 groups, one treated with SFU and the other with LV-LV + 5FU. Again, LV potentiated the effect of SFU. Also in a Colon 38 tumor which had developed resistance against SFU and which was reimplanted, LV potentiated the antitumor activity of SFU. Weight loss of the combination was slightly higher than for SFU alone. A moderate leukopenia (nadir 40 %) and mild anemia were observed, which were less than for SFU alone. The combination did not cause thrombocytopenia. In conclusion, LV can potentiate the therapeutic efficacy of SFU in murine colon carcinoma.

leucovorin / 5-fluorouracil / murine colon carcinoma

Résumé — Potentialisation in vivo du 5-fluorouracile par la leucovorine dans le carcinome colique murin.
Des résultats obtenus in vitro ont clairement montré que la leucovorine (LV) peut favoriser les effets inhibiteurs sur la croissance du 5-fluorouracile (SFU); en revanche, la potentialisation in vivo de l'effet anti-tumoral du SFU par la LV n'a pas encore été déterminée sur des modèles animaux. L'effet anti-tumoral et la toxicité de l'association LV-SFU ont été étudiés chez des souris porteurs de carcinomes coliques, colon 26 et colon 38. Les souris ont reçu une fois par semaine du SFU à la dose maximale tolérée (100 mg/kg) seul ou avec de la LV à des doses et selon des modalités différentes. Un prétreatment par la LV suivi 1 hr plus tard d'une deuxième injection de LV associée au SFU potentialise nettement l'effet antitumoral du SFU aussi bien dans l'une que dans l'autre lignée tumorale. Des résultats comparables ont été obtenus avec des doses totales de LV allant de 100 — 200 mg/kg. L'effet d'un traitement préalable par SFU a été étudié avec randomisation entre 2 groupes de souris de colon 38, les unes étant traitées par SFU, les autres par LV puis par LV + SFU. Là encore, la LV a potentialisé l'effet du SFU. De même, sur une tumeur colon 38 qui avait développé une résistance contre le SFU et avait été réimplantée, la LV a potentialisé l'action anti-tumorale du SFU. La perte de poids observée avec le traitement associé était légèrement plus importante qu'avec le SFU seul. Une leucopénie modérée (supérieure ou égale à 40 %) ainsi qu'une légère anémie ont été observées, mais ces anomalies étaient moins marquées qu'avec le SFU seul. L'association n'a pas provoqué de thrombopénie. En conclusion, la leucovorine est capable de renforcer les propriétés thérapeutiques du SFU sur le carcinome colique murin.

leucovorine / 5-fluorouracile / carcinome colique murin

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Introduction

5-Fluorouracil (5FU) is still considered as the standard therapy in the treatment of advanced colorectal cancer [15]. However, objective response rates are only 10-20%. The therapeutic efficacy of 5FU might be enhanced by biochemical modulation, which implies the administration of an agent which can interfere with the mechanism of action of an antitumor agent in order to selectively increase its antitumor properties [11, 25]. Leucovorin (5-formyl-tetrahydrofolate; LV) has attracted special interest as a modulating agent in clinical oncology.

5FU has to be converted to fluoro-2-deoxy-5'-monophosphate (FdUMP) [25], which forms a covalently bound ternary complex with 5,10-methylene-tetrahydrofolate (CH₂THF) and thymidylate synthase (TS) leading to inhibition of dTMP synthesis and subsequently DNA synthesis [25]. In the absence of CH₂THF, a binary complex is formed and then FdUMP acts as a weak inhibitor of TS [26]. The stability of the ternary complex is highly dependent on the availability of CH₂THF [5, 6, 29]. In vitro studies with several 5FU resistant human tumor xenografts demonstrated that maximal binding of FdUMP could only be achieved with excess concentrations of CH₂THF [5]. Addition of LV enhanced the growth inhibition induced by 5FU in several tumor cell lines [2, 8, 14]. 5FU cytotoxicity may also be related to its incorporation into RNA [20, 21, 25]. It has been suggested that 5FU might shift from its RNA action to the inhibition of TS when LV is added [2].

Preclinical in vivo studies with L1210 leukemia failed to demonstrate a potentiation of 5FU activity by LV [9]. These negative results might be related to the use of either the L1210 system or the schedules used.

Based on the preclinical in vitro results, several clinical trials have been initiated. In patients not pretreated with 5FU, consistently higher but variable response rates have been observed in comparison to 5FU alone [3, 10, 12, 24]; however, a large variety of schedules and doses of LV and 5FU have been used [3]. For patients pretreated with 5FU, who appeared to be resistant to the treatment, less favorable results were observed [27], although this might be related to dose and schedule. The present study describes the potentiation of 5FU by LV in a relatively resistant, a sensitive tumor and a 5FU-pretreated tumor. The selectivity of the combination was also evaluated by documenting the toxicity of the combination.

Materials and Methods

5FU and LV (Lederle-Cyanamid, Etten-Leur, The Netherlands) were prepared as a 10 mg/ml solution. Total injection volume was between 0.15 - 0.30 ml per mouse. The murine tumors Colon 26 (undifferentiated carcinoma with local fibrosarcoma) and Colon 38 (adenocarcinoma) were maintained in female Balb-C and C57B1/6 mice, respectively, as previously described [22, 23]. Mice were randomized in groups of 6, including a control group. Treatment was started when tumor volume was between 50 - 200 mm³ and was performed between 2 - 4 p.m. to prevent diurnal variations in the efficacy of 5FU [22]. Tumor measurements were performed by caliper determinations twice weekly and tumor volume was calculated (length × width × height × 0.5). Growth delay factor (GDF) was determined by the formula: \( \delta = \frac{TD(t)}{TD(c)} \) where TD(t) and TD(c) are the mean tumor doubling times of treated and control non-treated mice.

Hematological toxicity was evaluated by measurement of the hematocrit, leukocyte and thrombocyte counts in non-tumor bearing C57B1/6 mice. Student's t-test was used for statistical analysis of the results.

Results

5FU was tested at its maximum tolerated dose (MTD) for 4 weekly injections, 100 mg/kg [18, 22, 23]. The overall weight loss of 5 - 15% was taken as a parameter for the MTD. In combination with LV the same dose of 5FU was used. Colon 38 is a 5FU-sensitive tumor, while Colon 26 was considered to be resistant, since the GDF was usually lower than 1. Details about the optimization of the schedule of LV have been described elsewhere [18]. Simultaneous administration of LV and 5FU did not improve the antitumor activity of 5FU; a pretreatment with LV was required. The best results in both Colon 26 and Colon 38 (Fig. 1) were obtained when mice were treated with LV 1 hr before and together with 5FU (LV-LV + 5FU).

For Colon 26, administration of various doses of LV as a single bolus administration or divided in several injections were studied. A total dose of 200 mg/kg did potentiate the effect of 5FU alone, but the effects were not better than those observed with LV at 100 mg/kg. Delayed administration of LV did not improve the antitumor activity observed with the LV-LV + 5FU schedule (Table I).

Clinically it is of interest to know whether acquired resistance against 5FU can be overcome.
by treatment with the combination of LV and 5FU. For this reason we treated C57Bl/6 mice bearing Colon 38 for 4 weeks with 5FU and randomized them in 2 groups, one being treated for 4 additional weeks with 5FU and the other with the active schedule, LV-LV+5FU. Another group of mice was treated for 8 weeks with the LV-LV+5FU combination. The latter gave significantly better growth delay than the 2 other schedules (Fig. 2). Treatment of the 5FU pretreated group with LV-LV+5FU also resulted in a better antitumor effect than 5FU when compared from the day of randomization. Furthermore, 2 complete responders in the 5FU group recurred but 3 complete responders in the LV-LV+5FU did not. Similar results were obtained in a separate experiment (data not shown).
Table I. Summary of the results of various combinations of 5FU and LV.

<table>
<thead>
<tr>
<th>Tumor : Schedule-dose (a)</th>
<th>Weight loss (b)</th>
<th>GDF (c)</th>
<th>P (d)</th>
<th>P (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon 26: 5FU</td>
<td>9.2; 4.6; 8.7</td>
<td>0.9; 0.5; 1.3</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>LV - 5FU (100 - 100)</td>
<td>5.3</td>
<td>0.9</td>
<td>&lt; 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>LV - LV + 5FU (50 - 50 + 100)</td>
<td>8.4</td>
<td>2.0</td>
<td>&lt; 0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>LV - LV + 5FU (100 - 100 + 100)</td>
<td>6.0; 6.4; 7.3</td>
<td>2.9; &gt;3; 2.7</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>LV - LV + 5FU - LV (50 - 50 + 100 - 50)</td>
<td>5.8; 7.7</td>
<td>2.9; &gt;4</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>LV + 5FU - LV (50 - 50 + 50)</td>
<td>6.8</td>
<td>1.7</td>
<td>&lt; 0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>Colon 38: 5FU (100)</td>
<td>4.3*</td>
<td>1.8</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>LV - 5FU (100 - 100)</td>
<td>5.7</td>
<td>1.9</td>
<td>&lt; 0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>LV - LV + 5FU (50 - 50 + 100)</td>
<td>8.7*</td>
<td>3.1</td>
<td>&lt; 0.01</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>Colon 38 (long-term): 5FU (100) (8 wks)</td>
<td>4.7</td>
<td>5.9</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>LV - LV + 5FU (50 - 50 + 100) (8 wks)</td>
<td>4.1</td>
<td>8.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>5FU (100) (&gt; d 24)</td>
<td>1.2 *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV - LV + 5FU (50 - 50 + 100) (&gt; d 24)</td>
<td>2.1 *</td>
<td></td>
<td></td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Colon “38R”: 5FU (100)</td>
<td>3.7</td>
<td>2.8</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>LV - LV + 5FU (50 - 50 + 100)</td>
<td>3.8</td>
<td>4.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.02</td>
</tr>
</tbody>
</table>

All data are from separate experiments.
(a) The dashes indicate a time difference of 1 hr; doses are in mg/kg.
(b) Mean % percent weight loss at 1 day after treatment. *, weight loss between these 2 groups was significantly different at the level 0.02 < P < 0.05.
(c) GDF was calculated from TD from treated and non-treated tumors. The means TD of non-treated tumors were 3.15 for Colon 26, 6.85 for Colon 38 and 6.05 for the “5FU-induced” resistant Colon “38R”.
(d) P-value of the statistical evaluation of the comparison of the TD of treated and non-treated tumors.
(e) P-value of the comparison of the TD of the various LV - 5FU schedules with the 5FU schedule within the same experiment; n.s. not significant.

* TD was calculated using day 24 as day 0. Starting that day the 5FU-treated mice were randomized in 2 groups, one received 5FU for an additional 4 weeks, the other received LV - LV + 5FU for 4 weeks. The TDs from these groups were significantly different at the level 0.02 < P < 0.05.

Acquired resistance against 5FU was also studied in Colon 38 tumors which showed progression during a 10-week single agent 5FU treatment. One of these tumors, called Colon “38R”, was reimplanted in healthy C57Bl/6 mice, and treated either with 5FU or with the LV-LV+5FU combination. This Colon “38R” was no longer resistant and showed a significant tumor growth delay with both 5FU alone and LV-LV+5FU (Table I). Treatment with the combination was slightly better than with 5FU alone. However, no complete regressions were observed with both schedules.

Weight loss due to treatment could not be evaluated properly in Colon 26-bearing mice because these mice suffered weight loss due to toxicity of the tumor (~ 15% in 10 days). Treatment partially prevented this weight loss (5 - 10% weight loss in 10 days). Therefore only the weight loss after one day is given, which is considered to be directly related to treatment. A moderate weight loss was observed in Colon 38-bearing mice treated with 5FU alone, which was significantly higher in the LV-LV+5FU regimen (Table I). Mice recovered before the next treatment.

Treatment with the LV-LV+5FU combination showed a moderate leukopenia (nadir at day 11), and a significant fall in the hematocrit values. Single agent treatment with 5FU caused a more severe leukopenia and anemia (Fig. 3). No thrombocytopenia was observed with both 5FU alone and LV-LV+5FU, but a rebound was observed when treatment was discontinued. This rebound was higher in the single agent 5FU regimen (data not shown).
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Discussion

Our results show that LV can potentiate the antitumor effect of 5FU against solid murine colon carcinoma. The effect is highly dependent on scheduling; simultaneous and delayed administration of LV did not enhance the efficacy of 5FU, but LV given before and together with 5FU showed a significantly better effect than 5FU alone. Furthermore, in 5FU-pretreated mice, this schedule was also more active than 5FU. Toxicity was mild and not more serious than with 5FU alone.

Klubes et al. [9] have reported a negative study on the LV-5FU combination in L1210 leukemia-bearing mice. A rather low dose of LV was administered after 5FU, while antitumor activity could only be evaluated by differences in life span. Another negative study has been published recently [13] with murine breast tumors. Although higher LV doses were used, they were administered 1 hr before and at several time-points after 5FU. It seems likely that the potentiation of 5FU by LV is schedule-dependent, since in our study delayed LV did not potentiate the antitumor effect of 5FU.

Mini et al. [14] also observed a sequence dependence in the potentiating effect of 2'-deoxy-5-fluorouridine by LV using leukemia cells in vitro. Delayed LV did not potentiate. This sequence dependence may be related to the mechanism of inhibition of TS and the formation of mono- and polyglutamates of CH₃THF. In vitro reduced folates were rapidly lost from cells to the medium by an efflux mechanism when cells were preloaded with folylpolyglutamates [19]. However, folylpolyglutamates can accumulate intracellularly above the level of reduced folates of equilibrium with the extracellular medium [16]. Recently, it was demonstrated for Colon 38 that LV increased intratumoral folate pools [17]; in human xenografts the highest level of reduced folates was observed 1 hr after bolus LV administration [7] with an increase of polyglutamates. So at injection of 5FU conditions are optimal to permit binding of FdUMP to TS. High 5FU levels (> 50 μM) are present in Colon 26 and Colon 38 (Peters et al., unpublished data) for at least 2 hr. The second injection of LV together with 5FU would lead to formation of new folylmono- and polyglutamates and maintain optimal conditions to permit binding of FdUMP to TS.

Our studies showed an enhanced antitumor effect both in a 5FU-sensitive tumor line and a line with intrinsic resistance. This advantage was also observed when tumor resistance was induced by long-term treatment with 5FU. This means that 5FU-induced resistance in these tumors can be overcome by subsequent treatment with LV-LV+5FU. However, the difference between 5FU and LV-LV+5FU (Fig. 2) was less than in non-5FU pretreated tumors. This might be related...
to several phenomena [25], such as a low FdUMP/dUMP ratio, amplification of the target enzyme TS, alterations in the activity of key enzymes in the activation mechanisms, or alterations in folate metabolism. Further biochemical studies are required to define the mechanism.

Results concerning clinical trials are encouraging, despite the large variation in doses and schedules. Phase III trials showed significantly higher response rates for the combination compared with 5FU as a single agent [1, 24]. Interestingly, these high response rates were obtained when LV was administered at least 1 hr before 5FU, in agreement with our findings. In patients pretreated with 5FU no responses were observed [27], possibly due to development of resistance. However, the use of very high LV doses (500 mg/m²) in a weekly schedule resulted in better results [4]. Although we did not observe an effect of LV dosing in the antitumor activity with Colon 26, very high doses might also further improve the therapeutic efficacy in our model.

Overall toxicity of 5FU was not enhanced by the addition of LV. But a shift in the nature of toxicity was observed. Hematological toxicity was even less than with 5FU, which is in agreement with data observed in patients [1] treated with LV and 5FU in a weekly schedule. Recently Wright et al. [28] demonstrated that LV did not increase CH₃THF levels in bone marrow of mice, which might explain the lack of enhanced myeloid toxicity. Gastrointestinal toxicity in patients is dose limiting. In C57Bl mice we observed a higher weight loss in the active LV-LV+5FU schedule. Furthermore, in the long-term schedule (Fig. 2), toxic deaths following severe weight loss have been observed in 2 separate experiments in contrast to single agent 5FU.

In summary, LV administered before 5FU potentiated the antitumor activity of 5FU in murine colon carcinoma. The best results were obtained when LV was given twice, 1 hr before and together with 5FU. Toxicity was mild. These enhanced effects were also seen when tumor resistance was induced by pretreatment with 5FU.

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References


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